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Inhibitory effects of cardols and related compounds on superoxide anion generation by xanthine oxidase

Noriyoshi Masuoka [a,*], Ken-ichi Nihei [b], Ayami Maeta [a], Yoshiro Yamagiwa [c], Isao Kubo [b]

[a] Department of Life Science, Okayama University of Science, 1-1 Ridai-Cho, Okayama 700-0005, Japan
[b] Department of Environmental Science, Policy and Management, University of California, Berkeley, CA 94720-3114, USA
[c] Department of Chemistry, Kinki University, Osaka 577-8502, Japan

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ABSTRACT

5-Pentadecatrienylresorcinol, isolated from cashew nuts and commonly known as cardol ($C_{15:3}$), prevented the generation of superoxide radicals catalysed by xanthine oxidase without the inhibition of uric acid formation. The inhibition kinetics did not follow the Michelis–Menten equation, but instead followed the Hill equation. Cardol ($C_{10:0}$) also inhibited superoxide anion generation, but resorcinol and cardol ($C_{5:0}$) did not inhibit superoxide anion generation. The related compounds 3,5-dihydroxyphenyl alkanoates and alkyl 2,4-dihydroxybenzoates, had more than a C9 chain, cooperatively inhibited but alkyl 3,5-dihydroxybenzoates, regardless of their alkyl chain length, did not inhibit the superoxide anion generation. These results suggested that specific inhibitors for superoxide anion generation catalysed by xanthine oxidase consisted of an electron-rich resorcinol group and an alkyl chain having longer than C9 chain.

1. Introduction

Xanthine oxidase (EC 1.1.3.22), a molybdenum-containing enzyme, catalyses the oxidation of hypoxanthine to xanthine and ultimately to uric acid. The accumulation of uric acid leads to hyperuricaemia and gout (Hatano et al., 1990; Nakanishi et al., 1990), therefore xanthine oxidase inhibitors may serve as therapeutic agents for these conditions. Xanthine oxidase also generates a superoxide anion, and excess superoxide anion generation leads to peroxidative damage in cells (Fong, McCay, Poyer, Keele, & Misra, 1973; McCord, 1985). Intake of xanthine oxidase inhibitors from foods may be useful to prevent postischemic injury. In our continuing investigation to understand the functions of xanthine oxidase inhibitors, the balance of hydrophilic and hydrophobic moieties of molecules is associated with the inhibitory activity (Masuoka & Kubo, 2004; Masuoka, Nihei, & Kubo, 2006; Masuoka et al., 2012). 1, 1-Diphenyl-2-*p*-picryhydrazyl (DPPH) scavenging activity of antioxidants is attributed to their conjugated endiol structures, and the activity indicates a reduction activity. Flavonoids are essentially competitive inhibitors for uric acid formation catalysed by xanthine oxidase. Some flavonoids, which have DPPH scavenging activity, are able to strongly reduce xanthine oxidase molecules to suppress superoxide anion generation (Masuoka, Matsuda, & Kubo, 2012). Finally it has become evident that the reaction of xanthine oxidase with inhibitors consisted of inhibition of uric acid formation, reduction reactions of the xanthine oxidase molecule and radical scavenging reactions. The active sites of uric acid formation catalysed by xanthine oxidase and the superoxide anion generation are different. That is, an inhibitor which binds the xanthine binding site in xanthine oxidase inhibits the uric acid formation by xanthine oxidase. An inhibitor of superoxide anion generation binds to other sites, and this binding leads to the inhibition of superoxide anion generation or reduction of the enzyme molecules to catalyse hydrogen peroxide formation. Therefore, as xanthine oxidase inhibitors are able to multifunction, it is necessary to distinguish each function and to find specific inhibitor.

Anacardic acids ($C_{15:3}$) (**1**) in Fig. 1, isolated from the cashew *Anacardium occidentale* (*Anacardiaceae*) (Kubo, Komatsu, & Ochi, 1986), had no DPPH activity and cooperatively bound to xanthine binding site to inhibit both uric acid formation and superoxide anion generation (Masuoka & Kubo, 2004). As cardol ($C_{15:3}$) (**2**), 5-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]resorcinol, isolated from the cashew, was not an inhibitor of uric acid formation but did inhibit the generation of the superoxide anion, we suggested that cardol was a specific inhibitor for superoxide anion generation catalysed by xanthine oxidase (Masuoka et al., 2012). However, it is still unclear about the specific character of cardols. In the current study, prepared cardol ($C_{10:0}$) (**3**) and ($C_{5:0}$) (**4**) and related compounds (**7**–**15**) were examined and their effects on the xanthine oxidase

* Corresponding author. Tel./fax: +81 86 256 9593.
E-mail address: masuokan@dls.ous.ac.jp (N. Masuoka).

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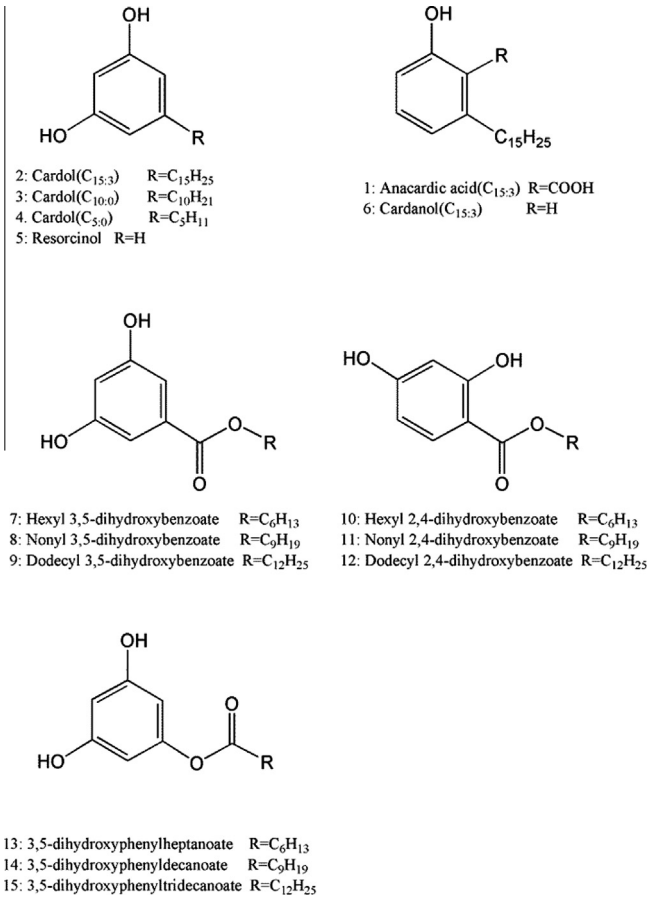

Fig. 1. Cardols and related compounds.

reaction to study the function of the hydrophobic head portion and hydrophilic tail portion of cardols.

2. Materials and methods

2.1. Chemicals

Cardol ($C_{15:3}$) (**2**), anacardic acid ($C_{15:3}$) (**1**) and cardanol ($C_{15:3}$) (**6**) used for the assay were previously isolated from the cashew nut shell oil and were re-purified by recycle HPLC (R-HPLC) using an ODS C_{18} column. Cardols (**3, 4**) possessing different alkyl side chains were synthesized from 3,5-dimethoxybenzaldehyde via a Wittig reaction. Xanthine oxidase, DPPH, EDTA and resorcinol were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI). Cardol related derivatives, such as alkyl 3,5-dihydroxybenzoates (**7**–**9**), alkyl 2, 4-dihydroxybenzoates (**10**–**12**) and 3,5-dihydroxyphenyl alkanoates (**13**–**15**), were synthesized as follows. First, hexyl, nonyl and dodecyl 3,5-dihydroxybenzoates (**7**–**9**) were synthesized by a two-step procedure. The corresponding dibenzyloxybenzoic acids were obtained first by a method previously reported (Nihei, Nihei, & Kubo, 2003). Then, alkyl 3,5-dihydroxybenzoates, were obtained in high yield by a two step procedure, a Mitsunobu reaction the first step, followed by hydrogenation to remove the protecting group. Second, the same alkyl (hexyl, nonyl and dodecyl) 2,4-dihydroxybenzoates (**10**–**12**) were synthesized in a similar manner using 2,4-dibenzyloxybezoic acid as a starting material. Third, 3,5-dihydroxyphenyl (heptanoate, decanoate and tridecanoate) alkanoates (**13**–**15**) were readily prepared in one step from phloroglucinol and the corresponding carboxylic acid using DCC as a coupling reagent.

2.2. Preparation of sample solution

Compounds were dissolved in dimethyl sulfoxide (DMSO), to a concentration of 10 mM, which was used for the experiments.

2.3. Assay of uric acid generated by xanthine oxidase

The xanthine oxidase (EC 1.1.3.22, Grade IV) used for the bioassay was purchased from Sigma Chemical Co. The reaction mixture consisted of 2.76 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml of 10 mM xanthine and 0.06 ml of the sample solution (dissolved in DMSO). The reaction was started by the addition of 0.12 ml of xanthine oxidase (0.04 units), and the absorbance at 293 nm was recorded for 60 s. A control experiment carried out by replacing the sample solution with the same amount of DMSO. The reaction rate was calculated from the proportional increase in absorbance.

2.4. Assay of superoxide anion generated by xanthine oxidase

In the reaction, the superoxide anion generated by the enzyme reduces nitroblue tetrazolium to a blue formazan. The absorbance of the formazan produced was determined at 560 nm. The reaction mixture consisted of 2.7 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml of 10 mM xanthine and 0.03 ml of 0.5% bovine serum albumin, 0.03 ml of 2.5 mM nitroblue tetrazolium and 0.06 ml of the sample solution (dissolved in DMSO). To the mixture at 25 °C, 0.12 ml of xanthine oxidase (0.04 units) was added, and the absorbance at 560 nm was recorded for 60 s (by formation of blue formazan) (Masuoka & Kubo, 2004). A control experiment was carried out by replacing the sample solution with the same amount of DMSO.

2.5. Radical scavenging activity on DPPH

First, 1 ml of 100 mM acetate buffer (pH 5.5), 1.87 ml of ethanol and 0.1 ml of ethanolic solution of 3 mM DPPH were put into a test tube. Then, 0.03 ml of the sample solution (dissolved in DMSO) was added to the tube and incubated at 25 °C for 20 min. The absorbance at 517 nm (DPPH, $\varepsilon = 8.32 \times 10^3$) was recorded. As a control, 0.03 ml of DMSO was added to the tube. The scavenging activity was calculated from the decrease in absorbance and expressed as the number of scavenged DPPH molecules per each sample molecule.

2.6. Radical scavenging activity for the O_2^- generated by the PMS-NADH system

The superoxide anion was generated nonenzymatically with a PMS-NADH system. The reaction mixture (final volume was 3.0 ml) containing 25 μM NBT, 150 μg of BSA, 78 μM NADH and 0.06 ml of sample solution in 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0) was prepared and incubated at 25 °C for 3 min. Then, 0.03 ml of 155 μM PMS was added to start the reaction and the absorbance at 560 nm was recorded for 60 s (Nishikimi, Rao, & Yagi, 1972). As the control, 0.06 ml of DMSO was used. The reaction rate was calculated from the proportional increase of absorbance, and the scavenging activity of the sample was expressed as the inhibition percentage.

2.7. Assay and data analysis

Each assay was performed at least in triplicate in separate experiments, and the analysis was performed using Sigma plot 2001 (SPSS Inc., Chicago, IL). The inhibition mode and kinetic parameters were analysed with Enzyme Kinetics Module 1.1 (SPSS